Determination of the Volume Changes for Pressure-Induced Transitions of Apomyoglobin between the Native, Molten Globule, and Unfolded States

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ABSTRACT The volume change for the transition from the native state of horse heart apomyoglobin to a pressure-induced intermediate with fluorescence properties similar to those of the well-established molten globule or I form was measured to be −70 ml/mol. Complete unfolding of the protein by pressure at pH 4.2 revealed an upper limit for the unfolding of the intermediate of −61 ml/mol. At 0.3 M guanidine hydrochloride, the entire transition from native to molten globule to unfolded state was observed in the available pressure range below 2.5 kbar. The volume change for the N→I transition is relatively large and does not correlate well with the changes in relative hydration for these transitions derived from measurements of the changes in heat capacity, consistent with the previously observed lack of correlation between the *m*-value for denaturant-induced transitions and the measured volume change of unfolding for cooperativity mutants of staphylococcal nuclease (Frye et al. 1996. *Biochemistry.* 35:10234–10239). Our results support the hypothesis that the volume change associated with the hydration of protein surface upon unfolding may involve both positive and negative underlying contributions that effectively cancel, and that the measured volume changes for protein structural transitions arise from another source, perhaps the elimination of void volume due to packing defects in the structured chains.

INTRODUCTION

Apomyoglobin is known to populate conformations of intermediate stability under conditions of low pH, or in the presence of relatively low concentrations of denaturants (Kirby and Steiner, 1970; Balestrieri et al., 1976; Goto and Fink, 1990; Griko et al., 1988; Hagihara et al., 1993; Hughson et al., 1990; Barrick and Baldwin, 1993). The structure and thermodynamic properties of the most stable of these intermediates, termed the I state or the molten globule state, have been examined by a wide variety of physical techniques. Circular dichroism has demonstrated that this state retains a significant degree of helicity relative to the unfolded state, U (Hughson et al., 1990). NMR studies have identified three stable helices, A, G, and H, which form a structured core (Hughson et al., 1990), which is somewhat more loosely packed than in the native form (Hughson et al., 1991; Kay and Baldwin, 1996). The radius of gyration is only slightly larger than native apomyoglobin (Gast et al., 1994; Kataoka et al., 1995), and fluorescence studies have shown that the degree of exposure of the two tryptophan residues found at positions 7 and 14 in the A helix is intermediate between the native and unfolded forms (Irace et al., 1981; Kirby and Steiner, 1970). The cooperativity or *m*-values for the urea unfolding transitions of apoMb from the native to the I state and from the I state to the unfolded state are quite similar, indicating (like the tryptophan spectra) exposure of similar amounts of surface area for these two transitions (Barrick and Baldwin, 1993). In contrast, calorimetric studies have shown that the molten globule state exhibits a heat capacity similar to that of the native state and significantly lower than that of the unfolded state, indicative of a much lower degree of hydration (Griko and Privalov, 1994).

Perturbation of native protein structure by the application of high hydrostatic pressure can also yield information concerning the physical properties of the various states that may be populated. Pressure leads to the destabilization of native protein structure due to Le Chatelier's principle. The application of pressure displaces a chemical equilibrium in the direction in which the system volume is smallest, or $d\Delta G/dp = \Delta V$. The volume of the system refers to the total volume occupied by the protein and the solvent, and this volume has been shown to be smaller for the unfolded states of proteins compared to their native states. Based on model compound studies, the factors that contribute to the measured overall volume change for the unfolding of proteins include the electrostriction of charged and polar side chains and hydrophobic hydration (Kauzmann, 1959). In addition, the elimination of void volumes within the folded structure also contributes to an overall decrease in system volume upon unfolding of the protein (Weber and Drickamer, 1983). Although the measured volume changes for protein unfolding are almost always negative, they are quite small in absolute value, between 0.5% and 2% of the total volume of the protein. This has perplexed protein chemists for decades (Brandts, 1969; Brandts et al., 1970; Dill, 1990; Zipp and Kauzmann, 1973). The large negative volume change expected for hydrophobic hydration based on model

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system studies may be a severe overestimate, given that the specific volume of hydrophobic compounds in pure liquid is much larger than that of hydrophobic residues in the interior of proteins. Moreover, a significant positive contribution to ΔV_u has recently been proposed to arise from the thermal volume occupied by the exposed residues (Chalikian and Breslauer, 1996). Others have suggested that the contribution of hydrophobic hydration is positive rather than negative (Bøje and Hvidt, 1972; Prehoda and Markley, 1996). Regardless of the underlying parameterization of the ΔV of unfolding, it is generally accepted that this volume change reflects an increased interaction between the polypeptide chain and the solvent (Chalikian and Breslauer, 1996; Mozhaev et al., 1996; Weber and Drickamer, 1983).

Upon application of pressure at neutral pH, apomyoglobin has been shown by fluorescence spectroscopy (Bismuto et al., 1996a,b; Prehoda et al., 1996) to populate an intermediate state with intrinsic fluorescence characteristics and 1,8-anilinonaphtalenesulfonate (ANS) binding properties similar to those observed for the molten globule intermediate obtained at pH 4.0 at atmospheric pressure. The high pressure (2.4 kbar) form of apomyoglobin at neutral pH has lost its ability to bind ANS, indicating disruption of the heme pocket by pressure. In the presence of a higher affinity fluorescence probe for the heme pocket, 2′-(*N,N*-dimethyl) amino-6-naphtoyl-4-transcyclohexanoic acid (DANCA), pressure results in a structural transition to a state with a 10-fold lower affinity for the probe, indicating that probe binding stabilizes the pocket against total disruption by pressure in this case (Sire et al., 1996). In the absence of a ligand for the heme pocket, pressure likely completely destroys the heme pocket integrity. At neutral pH, upon application of pressure, the intrinsic fluorescence of the two tryptophan residues in the A helix of apomyoglobin exhibits an increase in intensity and a shift to longer wavelengths characteristic of the molten globule intermediate (Bismuto et al., 1996b; this paper). In the studies presented below, our objective was to determine the volume changes associated with the transition from the native state of horse heart apomyoglobin to the molten globule intermediate and for the complete unfolding of the molten globule.

MATERIALS AND METHODS

Protein preparation

Horse skeletal muscle myoglobin was obtained from Sigma and was used without further purification. apoMb was prepared according to the Teale method (Teale, 1959) and lyophylized. The content of holo protein was determined at 409 nm ($\epsilon = 1.6 \times 10^5\ M^{-1}\ cm^{-1}$) and appeared to be ~1%. The solutions of apoMb were prepared from lyophylate at concentrations of ~30 μM; concentrations in solution were determined optically (Hitachi U3000) at 280 nm ($\epsilon = 1.43 \times 10^4\ M^{-1}\ cm^{-1}$) (Crumpton and Polson, 1965). All experiments were carried out at 21°C.

High-pressure data acquisition and analysis

High hydrostatic pressure was generated by a system similar to that described by Paladini and Weber (1981) in a Vascomax high-pressure cell. Tryptophan emission spectra of apoMb were collected in pressure steps of 100 bar. The excitation light source was a xenon arc lamp with the excitation monochromator set at 295 nm, with slits set for 16-nm bandpass widths. Tryptophan emission spectra were collected at the right angle setting with a PMT attached to scanning monochromator (ISS, Champaign, IL). Scan speed was 0.5–1 nm/s. Raw intensity values cannot be compared between perssure runs because of inevitable differences in the alignment of the high-pressure cell in the fluorometer. To eliminate photobleaching in the presence of trichloroacetate (TCA), the time base was reduced to 0.2 s/nm, and the wavelength range scanned was reduced to 310–390 nm. In addition, the shutter was closed immediately after the scan and only reopened immediately before the next scan. Under these conditions, no photobleaching was observed.

Spectra were converted to wavenumbers and the weighted average emission energy υ_g was calculated using the intensity F_i at each wavenumber υ_i and transformed to average wavelength λ_g:

$$\upsilon_g = \Sigma_i F_i \upsilon_i / \Sigma_i F_i \tag{1}$$

$$\lambda_g = (1/\upsilon g) \times 10^7 \tag{2}$$

Analysis of the curves of average emission wavelength versus pressure p was performed using the analysis program BIOEQS (Royer et al., 1991; Royer and Beechem, 1992; Royer, 1993), fitting the spectral center of mass data in terms of a two-state transition from the native to molten globule intermediate (N→I), or from the globule intermediate to the fully unfolded state (I→U) to yielded free energy ΔG and volume change ΔV for the transitions, as previously described (Vidugiris et al., 1995):

$$\begin{aligned} \Delta G &= -RT \ln K_{eq} \\ &= -RT \ln((\lambda g(f) - \lambda g(p))/(\lambda g(p) - \lambda g(u))) \end{aligned} \tag{3}$$

and

$$d\Delta G/dp = \Delta V \tag{4}$$

where $\lambda g(n)$, $\lambda g(u)$, and $\lambda g(p)$ correspond to the average emission wavelengths of the native and intermediate states and that at pressure p in the case of the N→I transition, and to the average emission wavelength of the intermediate and unfolded states and that at pressure p for the I→U transition. The average emission wavelength pressure profile was quantum yield weighted by dividing the calculated ratio of folded/unfolded monomer by the relative quantum yield of the unfolded monomer. Rigorous confidence limit testing on the recovered parameters was carried out by performing a series of minimizations at all of the tested values of the parameter of interest, allowing all of the other parameters in the fit to vary. This method of rigorous confidence limit testing takes into account all of the correlations between parameters in the fit and provides much more realistic confidence limits than those typically derived from the diagonal elements of the correlation matrix.

RESULTS AND DISCUSSION

In Fig. 1 *a* are shown the normalized room temperature intrinsic fluorescence spectra of horse heart apomyoglobin taken between 310 nm and 390 nm at pH 6.0 and pH 4.2 in bis-Tris buffer. It can be seen that at the lower pH value, the fluorescence spectrum is shifted to longer wavelength, indicating a greater degree of exposure to solvent of the intrinsic tryptophan residues at positions 7 and 14 in the A helix. In fact, under these conditions, the protein populates to a significant degree, a partially folded intermediate termed the molten globule or I state (Barrick and Baldwin, 1993), the physical properties of which have been summarized above. In Fig. 1 *b* the normalized intrinsic fluores-

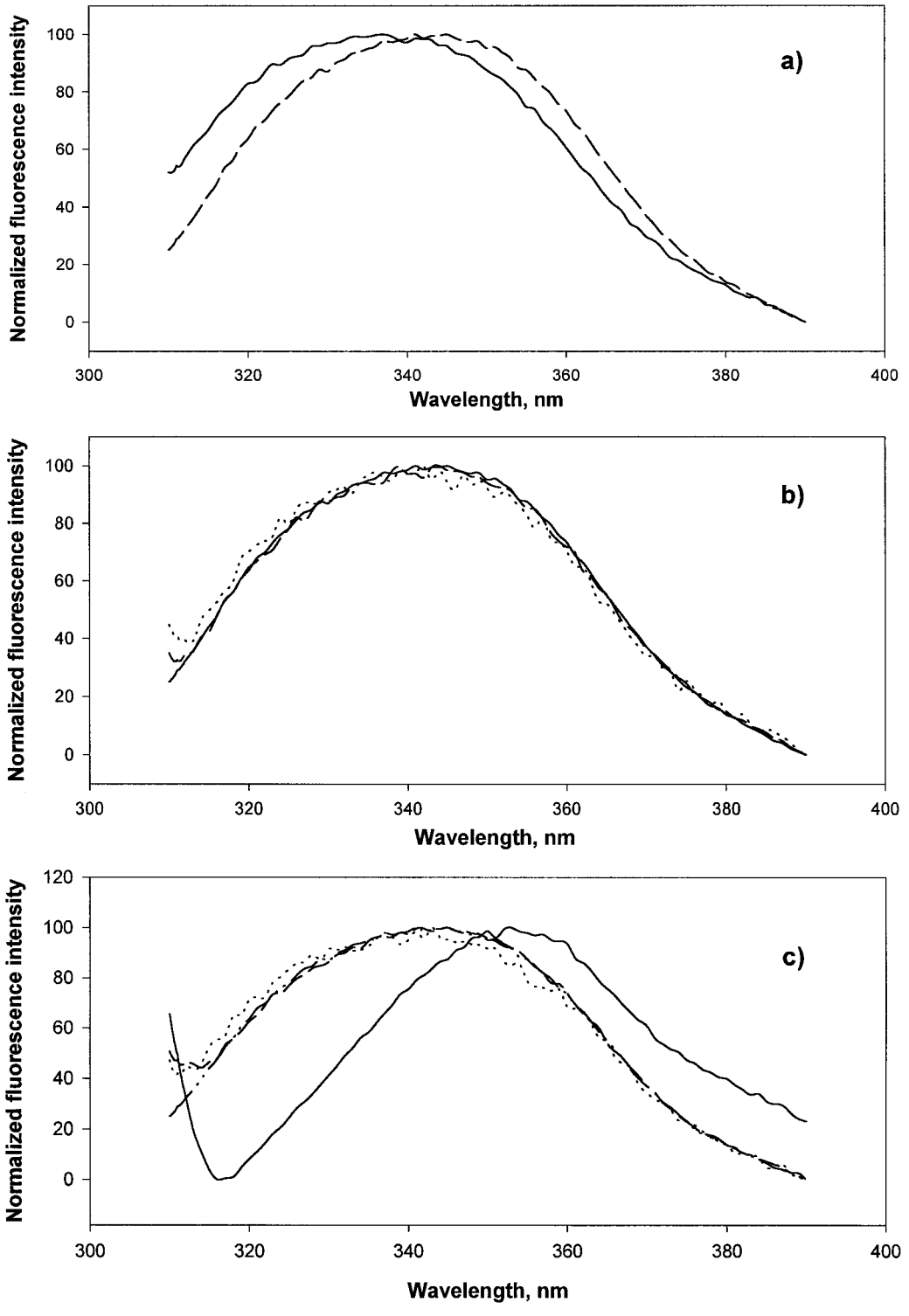

FIGURE 1 (*a*) Normalized room temperature intrinsic fluorescence spectra of horse heart apomyoglobin taken between 310 and 390 nm at pH 6.0 (——) and pH 4.2 (– – –) in bis-Tris buffer. (*b*) The normalized intrinsic fluorescence spectra at pH 4.2 in 10 mM sodium acetate buffer (——) and in the presence of either 20 mM trichloroacetate (TCA) (·····) or 0.4 M NaCl (– – –). (*c*) Normalized room temperature intrinsic fluorescence spectra of horse heart apomyoglobin taken between 310 and 390 nm at pH 4.2 in buffer (– ·· –) or at pH 2.6 in the presence of 0.4 M (– – –) NaCl or 20 mM TCA (Λ) and at pH 2.6 in the absence of any stabilizer (——).

cence spectra at pH 4.2 in buffer and in the presence of either 20 mM TCA or 0.4 M NaCl reveal that the exposure of the tryptophan residues under these conditions is quite similar. The emission maximum for the I state was found by Kirby and Steiner (1970) to be shifted to a longer wavelength by 5 nm compared to the native state, and was shifted

4 nm to a shorter wavelength compared to the unfolded state. Thus the transition from N→I results in a 56% change in the fluorescence emission wavelength, whereas the I→U transition involves the remaining 44%. Decreasing the pH from 6.0 to 4.2 at room temperature, we find that the shift is only 30% of the total shift to the unfolded state, indicating that the intermediate I state is not completely populated under these conditions.

The comparison of the spectra in Fig. 1 *c* at pH 4.2 in buffer or at pH 2.6 in the presence of 0.4 M NaCl or 20 mM TCA indicates that in terms of tryptophan exposure, these salt-stabilized acid structures are also similar to those obtained at pH 4.2. The addition of TCA to apomyoglobin solutions at pH 4.2 results in the stabilization of the A, G, and H helices and the addition of the B helix to the molten globule intermediate (Loh et al., 1995). Also shown in Fig. 1 *c* is the spectrum at pH 2.6 in the absence of any stabilizer. It can be seen that the acid denatured form exhibits a much more red-shifted spectrum than the intermediates obtained at pH 4 or in the presence of added salt or TCA.

Upon the application of hydrostatic pressure to native horse heart apomyoglobin at pH 6.0 in bis-Tris buffer, the spectrum shifts significantly to the red and increases in total intensity (Fig. 2). These changes in fluorescence properties are analogous to those observed as the pH is decreased to 4.2, where the protein populates to a significant degree the molten globule or I conformation. The plot of the average emission wavelength (calculated as described in Materials and Methods) as a function of pressure (Fig. 3, *circles*) reveals a sigmoidal profile suggestive of a structural transition induced by pressure. These results are similar to those obtained at neutral pH by Irace and co-workers (Bismuto et al., 1996b). The average emission wavelength at 2.5 kbar for the apomyoglobin at pH 6.0 (347.5 nm) is intermediate between the average emission energy of the native protein (342.7 nm) and that observed for the acid unfolded form at pH 2.6 (351.0 nm). Thus 59% of the total red shift observed upon the total unfolding of apomyoglobin is associated with the transition observed as the pressure is increased to 2.5 kbar at pH 6.0. This total change is quite similar to that observed as the molten globule is fully populated (56%; (Kirby and Steiner, 1970)). There is no obvious intermediate species in this sigmoidal profile. The analysis of the pressure dependence data obtained at pH 6.0 was therefore carried out in terms of a two-state unfolding transition from the native to the molten globule intermediate. The fit of the data to this simple model is seen to be very good (Fig. 3, *circles*), and thus analysis in terms of a more complicated model is clearly not warranted. The free energy for the N→I transition at atmospheric pressure was recovered to be $2.2 + 0.25/-0.07$ kcal/mol, and the volume change was $-70 + 0.21/-0.45$ ml/mol. This value for the volume change is in the range typically observed for the pressure unfolding of proteins and is comparable for example to that observed for the unfolding of staphylococcal nuclease by pressure (Vidugiris et al., 1995).

As the pH is decreased, apomyoglobin populates the molten globule intermediate (Hughson et al., 1990; Barrick and Baldwin, 1993). As discussed above, at pH 4.2 and room temperature, this intermediate is not 100% populated, and residual native state molecules are present. Upon the application of pressure to horse heart apomyoglobin at pH 4.2 in 10 mM sodium acetate buffer, the spectrum shifts further to the red, and the total intensity of emission decreases substantially. These spectral changes are analogous to those observed as the pH is decreased from 4.2 to 2.6, causing the complete unfolding of the molten globule, as well as that of the residual native state. The calculated average emission wavelength for apomyoglobin at pH 4.2 (Fig. 3, *squares*) shifts from near 345 nm at atmospheric

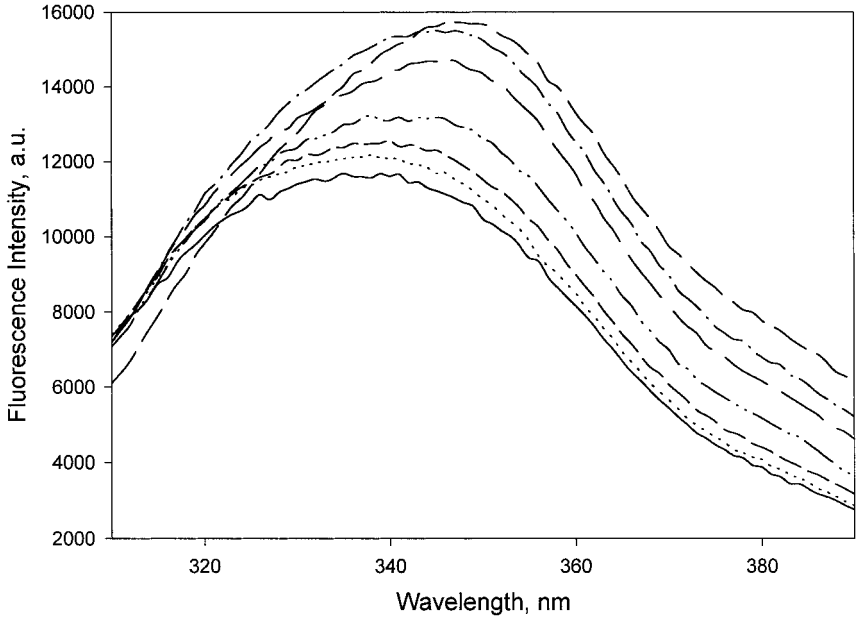

FIGURE 2 Dependence of the intrinsic fluorescence spectrum of native horse heart apomyoglobin at pH 6.0 in 10 mM bis-Tris buffer at 21°C on hydrostatic pressure. Hydrostatic pressure values: 0 bar (*solid line*); 150 bar (*dotted line*); 600 bar (*small dashed line*); 1000 Bar (*dash-double dotted line*); 1200 Bar (*large dashed line*); 1400 Bar (*dash-single dotted line*), and 2600 Bar (*medium dashed line*).

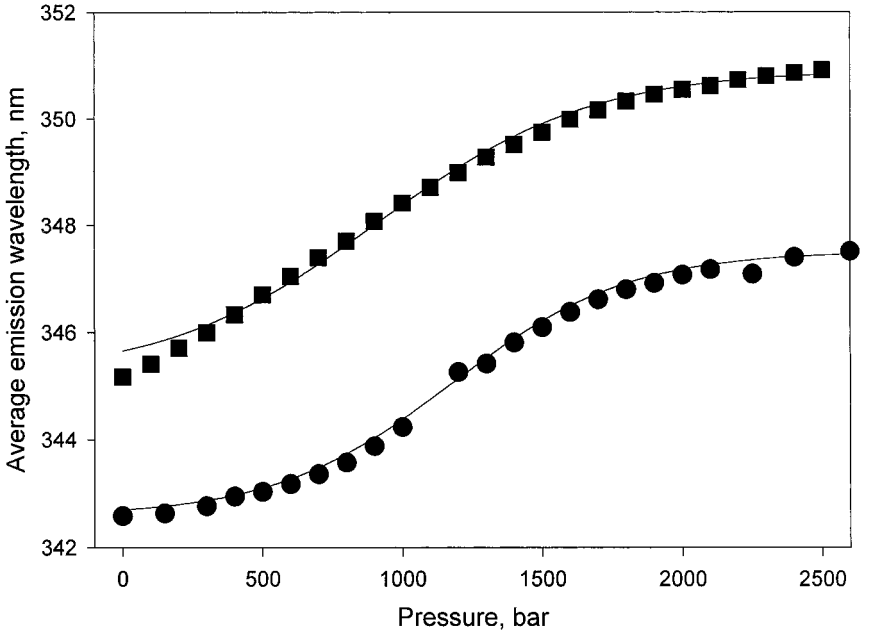

FIGURE 3 ●, Average emission wavelength (calculated as described in Materials and Methods) of native horse heart apomyoglobin at pH 6.0 in 10 mM bis-Tris buffer at 21°C as a function of pressure. ■, Calculated average emission wavelength for the molten globule state of horse heart apomyoglobin at pH 4.2, 10 mM sodium acetate, 21°C. Solid lines represent fits to the data points.

pressure in a sigmoidal fashion to 351 nm at 2.5 kbar, the value obtained for the fully acid-denatured form of the protein. The pH of the acetate buffer decreases slightly by ~0.4 pH units over this range as well (Zipp and Kauzmann, 1973), but the bulk of the observed spectral shift arises directly from the application of pressure. As discussed above, the protein does not populate the I state 100% at pH 4.2 and room temperature. However, if these data are analyzed in terms of a two-state transition from the molten globule intermediate to the fully unfolded state, one obtains a value of 1.4 + 0.24/−0.42 kcal/mol for the stability of the molten globule at atmospheric pressure and a volume change for the I→U transition of −61 +0.29/−7.6 ml/mol. It can be seen in Fig. 3 (*squares*) that the fit is not absolutely perfect, likely because of the residual population of the native state at low pressure. It is also possible that the transition from I to U is not a two-state transition. However, the simple two-state model describes the data reasonably well, and a more complex treatment would not yield well-determined parameters. The addition of either 20 mM TCA or 0.4 M NaCl to the apomyoglobin solution at pH 4.2 inhibited considerably the pressure-induced shift to a longer wavelength; the shift with pressure was less than half that observed in the absence of the stabilizers (345–347 nm), indicating protection against the pressure-induced unfolding of the intermediate by salt or TCA.

The addition of low concentrations of guanidine hydrochloride (GuHCl) below 0.5 M to solutions of apomyoglobin at pH 6.0 decreases the stability of the protein to denaturation by temperature or pH, but at room temperature and neutral pH, low concentrations of GuHCl do not significantly perturb the native structure. We have applied pressure to a solution of horse heart apomyoglobin at pH 6.0 in the presence of 0.3 M GuHCl. At pressures below 1 kbar, we observed a continuous increase in both the fluorescence intensity of the two intrinsic tryptophan residues and the average wavelength of their emissions (Fig. 4), as would be expected for the population of the molten globule state. As the pressure was further increased from 1 kbar to 2.5 kbar, the spectrum shifted further to the red, reaching 350 nm, a value similar to that obtained at pH 2.6 or at 2.5 kbar and pH 4.2. The intensity change observed between 1.0 and 2.5 kbar, however, reversed directions, as is observed for the unfolding of the molten globule state. Apparently, the addition of 0.3 M GuHCl destabilizes the apomyoglobin sufficiently that the entire three-state transition, N→I→U, such as that observed by Barrick and Baldwin (1993) as a function of pH, is observed in the presence of low concentrations of GuHCl in the pressure range below 2.5 kbar.

CONCLUSIONS

The pressure-dependent fluorescence profile of horse heart apomyoglobin at pH 6.0 presented a sigmoidal shift in the emission wavelength. The fluorescence spectral properties observed for this high-pressure intermediate obtained at pH 6.0 correspond to those observed previously at atmospheric pressure for the molten globule or I state of sperm whale apomyoglobin (Kirby and Steiner, 1970; Irace et al., 1981). It is thus highly likely that the application of pressure to native apomyoglobin results in the population of the molten globule (I) state, as previously suggested by Bismuto and co-workers (1996a,b). The fluorescence pressure profile fit quite well to a simple model assuming a two-state transition (N→I) from the native state to the molten globule intermediate. The volume change for this transition was recovered from the fit to be −70 ml/mol, and the stability was 2.2 kcal/mol. This change in volume is in the range typically found for the pressure dentauration of small proteins (e.g.,

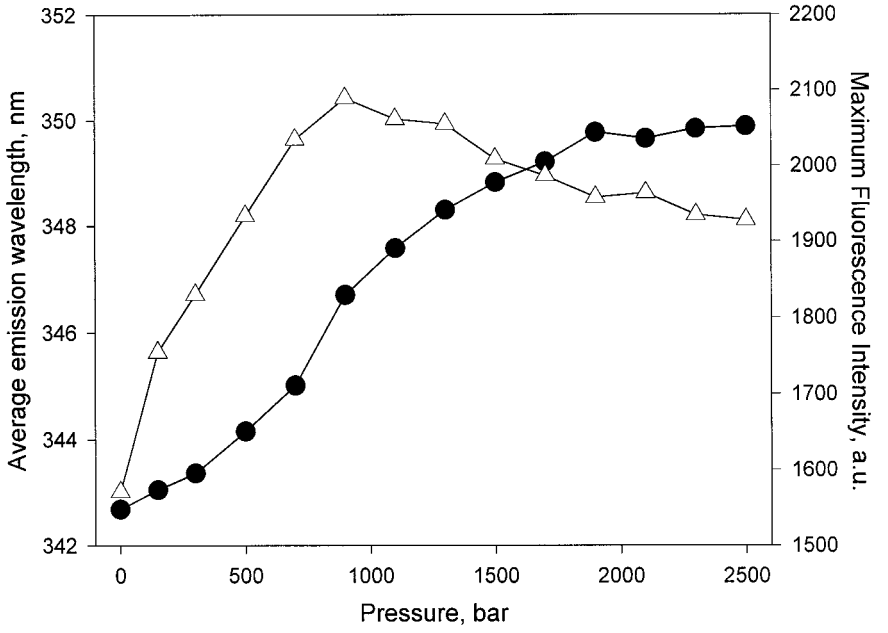

FIGURE 4 Effect of pressure on the average emission wavelength (●) and the maximum intensity of the intrinsic fluorescence (△) of native horse heart apomyoglobin at pH 6.0 in bis-Tris buffer at 21°C in the presence of 0.3 M guanidine hydrochloride.

Weber and Drickamer, 1983; Vidugiris et al., 1995). Pressurization of the intermediate form of apomyoglobin at pH 4.2 resulted in a transition to a state with fluorescence properties identical to those of the fully acid-denatured form of the protein. The volume change for unfolding of the intermediate is estimated to be near −61 ml/mol, while it exhibits marginal stability, −1.4 kcal/mol. Because the molten globule state is not 100% populated at room temperature and pH 4.2, analysis of the pressure data under these conditions in terms of a two-state transition from the I state to the U state yields approximate values for the free energy and volume changes of this transition. However, these are physically reasonable values. It is highly unlikely that the complete unfolding of apomyoglobin would greatly exceed 150 ml/mol (70 + 61 = 131) in absolute value, given the small size of the protein and the fact that the complete unfolding of metmyoglobin was accompanied by a volume change of about 100 ml/mol at pH 6.0 (Zipp and Kauzmann, 1973). The larger ΔV_u for apomyoglobin may arise from to an increase in the number of cavities in the structure of the apoprotein.

That pressure results in the formation of the molten globule intermediate of apomyoglobin is supported further by the results obtained at pH 6.0 in presence of 0.3 M GuHCl. Under these conditions, the changes in the fluorescence properties are clearly indicative of a three-state transition similar to that observed with pH (Barrick and Baldwin, 1993). The data clearly indicate the formation of an intermediate below 1 kbar, which is, in turn, unfolded as the pressure is increased to between 1 and 2.5 kbar. In fact, when the comparison of the effects of pressure on a variety of spectroscopic properties has been possible under pressure, it has been shown that the changes observed in fluorescence signals correlate well with those observed by NMR and infrared spectroscopy (Royer et al., 1993; Peng et al., 1994; Takeda et al., 1995; Panick et al., 1998) and indicate the loss of both secondary and tertiary structure under pressure. The significant stability of this intermediate under pressure is consistent with its equilibrium population under a variety of conditions, and supports the hypothesis (Barrick and Baldwin, 1993; Jennings and Wright, 1993) that this form of the protein represents an early intermediate on the folding pathway.

NMR studies of native sperm whale apomyoglobin (Eliezer and Wright, 1996) indicate that the EF loop, the F helix, the FG loop, and part of the G helix are disordered, because proton resonances in this region could not be assigned, because of conformational fluctuations, solvent exchange, or both. The remainder of the helices and loops in the native apomyoglobin, however, are well defined, suggestive of a structure close to that observed for the holoprotein. The MG intermediate form of apomyoglobin, on the other hand, exhibits proton exchange characteristics suggestive of structure only in the A, G, and H helices (Hughson et al., 1990). Moreover, the tertiary interactions in this intermediate are not entirely native-like, as evidenced by mutagenesis studies (Hughson et al., 1991; Kay and Baldwin, 1996) and the red-shifted tryptophan emission (Irace et al., 1981; Kirby and Steiner, 1970); this study).

Comparison of the heat capacities of the native, molten globule, and unfolded forms of sperm whale apomyoglobin (Griko and Privalov, 1994) reveals that only 10% of the total increase in heat capacity occurs in the N→I transition, whereas the remaining 90% is observed as the intermediate is unfolded. These data indicate that the bulk of the change in hydration (exposure of protein surface area to solvent) occurs upon the unfolding of the molten globule, and that this latter is relatively dry. The calorimetric observations appear in contrast to the very large value obtained in the present studies for the volume change for the N→I pressure

transition, which would indicate a significant change in the protein-solvent contact as the I state is populated. The value of −70 ml/mol obtained for the volume change in the pressure-induced N→I transition of horse heart apomyoglobin is comparable to other measured volume changes for complete unfolding of proteins. In fact, it is identical to that measured for the complete unfolding of staphylococcal nuclease, a protein of comparable molecular weight (Vidugiris et al., 1995), for which high-pressure small-angle x-ray scattering and Fourier transform infrared data indicate a degree of unfolding similar to that obtained with high concentrations of urea (Panick et al., 1998). In their determination of the parameters for the urea-induced unfolding of sperm whale apomyoglobin as a function of pH, Barrick and Baldwin (1993) report comparable *m*-values (which correlate with the degree of surface area exposure) for the N→I and I→U transitions. This result is consistent with those presented here indicating comparable magnitudes for the volume changes associated with the two transitions, although *m*-value and volume change do not necessarily correlate, probably because of compensating contributions to the volume change of hydrophobic and polar hydration (Frye et al., 1996).

One source of this apparent contradiction between the values of the ΔC_p and ΔV_u for the N→I transition of apomyoglobin may be that the I state obtained under pressure is more hydrated than that obtained by pH changes. However, we find the same changes in the intrinsic tryptophan emission spectrum for the pressure intermediate as are found for the I state when it is fully populated at atmospheric pressure. The tryptophan residues, as mentioned above, are found in the A helix, which remains ordered in the I state (Hughson et al., 1991), although less so in horse heart than in sperm whale apomyoglobin. Thus their degree of solvent exposure (as evidenced by their emission wavelength) provides a sound probe of I state structure. Moreover, as mentioned above, the *m*-values for the urea-induced unfolding at multiple pH values are comparable.

We are not the first to notice a lack of correlation between ΔC_p and ΔV. Zipp and Kauzmann (1973) remark, "It is clear that the simple hydrophobic model does not adequately explain the behavior of proteins under pressure." Klapper (1971) proposed, based on scaled particle theory calculations, that the small, negative volume changes observed upon protein unfolding arise from the fact that unlike apolar liquids, protein interiors are very tightly packed, and that thus exposure of hydrophobic groups should result in a positive volume change, which would be offset by a negative volume change upon exposure of polar and charged surfaces. Recently, our group has reported a lack of correlation between the amount of exposed surface area and the value of the volume change in unfolding of staphylococcal nuclease cooperativity mutants (Frye et al., 1996). We also suggested that the positive and negative contributions of hydration of exposed polypeptide surface area offset each other, and that therefore the overall measured volume change likely arises primarily from the elimination of packing defects. These packing defects have been calculated for a number of proteins to comprise 0–2% of the total protein volume (Rashin et al., 1986), similar to the observed values of the volume change of protein unfolding. Recent results on the pressure denaturation of cavity mutants of staphylococcal nuclease indicate that indeed, cavities provide a substantial contribution to the magnitude of the observed volume change for unfolding (K. J. Frye and C. A. Royer, submitted). Disruption of the heme pocket structure and the concomitant loss of heme pocket void volumes during the N→I transition I in apomyoglobin could account for the substantial decrease in system volume. This decrease in volume would not be correlated with an increase in hydration (as estimated by ΔC_p), because the heme pocket in native apomyoglobin is relatively well hydrated to begin with. However, loss of heme pocket structure could reveal binding sites for denaturant, and thus the N→I denaturation transition by urea would be cooperative, with a significant *m*-value, as previously demonstrated (Barrick and Baldwin, 1993).

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